Epidemiol. Infect. (2001), **127**, 1–5. © 2001 Cambridge University Press
DOI: 10.1017/S0950268801005763 Printed in the United Kingdom

Specific identification, grouping and differentiation of *Campylobacter jejuni* among thermophilic campylobacters using multiplex PCR

V. KOROLIK[1]*, D. T. FRIENDSHIP[2], T. PEDURU-HEWA[2], D. A. ALFREDSON[1], B. N. FRY[2] AND P. J. COLOE[2]

[1] *School of Health Science, Griffith University Gold Coast, QLD, Australia and* [2] *Applied Biology and Environmental Biology, RMIT University, Melbourne, VIC, Australia*

(*Accepted 23 March 2001*)

SUMMARY

Campylobacter species such as *C. jejuni* and *C. coli* are recognized as major causes of acute gastroenteritis world-wide. Although *C. jejuni* and *C. coli* are usually non-pathogenic in birds and animals, they cause enteric disease in humans and the source of infection is often the consumption of contaminated foodstuffs. In this paper we report on the development and use of a multiplex PCR of *C. jejuni* genomic DNA which yielded a PCR product with a unique polymorphic site that can be used to quickly and accurately identify and group *C. jejuni* isolates from any source including DNA, cell culture, skin washings and faecal samples. The test is simple and sensitive and can detect purified DNA from a single bacterium 10^2 cells from crude lysates, 10^3 cells in seeded faeces and 120 cells/ml of washing of 1 cm^2 skin fragments of chicken skin.

INTRODUCTION

Thermophilic *Campylobacter* species such as *C. jejuni* and *C. coli* are recognized as major causes of acute gastroenteritis world-wide in man and animals. Although *C. jejuni* and *C. coli* are usually non-pathogenic in birds and animals, the processing of poultry and other animal food products often leads to contamination of the end product, and the exposure of consumers to food poisoning organisms. This exposure, coupled with inadequate hygiene procedures that enable post-cooking contamination of products and/or the consumption of undercooked meats, can lead to the transfer of campylobacter disease to humans [1–3].

We have previously reported on the use of a specific DNA probe to differentiate between *Campylobacter* strains isolated from animals and humans by identification of a unique restriction endonuclease *Cla*I polymorphic site. The presence of this *Cla*I site in *C. jejuni* is characteristic of the majority of isolates from chickens, but is normally absent from human isolates [4]. In this study, a 747 bp sequence containing the polymorphic *Cla*I site, was utilized in combination with a sequence that identifies thermophilic campylobacters for the development of a rapid multiplex PCR based test. This test can simultaneously identify the medically important campylobacters, *C. jejuni* and *C. coli* differentiate between the two and also group the majority of *C. jejuni* strains into polymorphic groups. This PCR assay has been used to screen campylobacter isolates from human patients in Australia.

* Author for correspondence: School of Health Science, Gold Coast Campus, Griffith University, PMB 50 Gold Coast Mail centre, Queensland 9726, Australia.

METHODS

Bacterial strains and culture

Bacterial strains used to assay the multiplex PCR method were: from ACTC and NCTC culture collection: *Campylobacter hyoilei* CCUG 33450^T,

Campylobacter jejuni NCTC 11351^T, *Campylobacter jejuni* NCTC 11828^T (81116), *Campylobacter coli* NCTC 11366^T, *Campylobacter lari* NCTC 11352^T, *Campylobacter fetus* subsp. *fetus* NCTC 10842^T, *Campylobacter fetus* subsp. *veneralis* NCTC 10354^T, *Campylobacter mucosalis* NCTC 9350^T, *Campylobacter concisus* ATCC 51562^T, *Campylobacter sputorum* bv *faecalis* ATCC 33709^T, *Campylobacter sputorum* bv *bubulus* ATCC 33562^T; from – C. M. Patton, Atlanta, Georgia, USA: *Campylobacter upsaliensis D*54/7, *C. hyointestinalis* D1783, *Helicobacter cinaed* D1827, *Helicobacter fennelliae* D1833; from Monash University, Melbourne, Australia, culture collection: *Salmonella typhimurium*, 82/6915, *Salmonella enteritidis*, *Klebsiella pneumoniae* 180/2–6, *Proteus vulgaris* G701, *Yersinia enterocolitica*, from RMIT University, Melbourne, Australia, culture collection: *Escherichia coli* DHI, *Brachyspira hyodysenteriae*, 4/G131. Campylobacter isolates were from human patients from states of Victoria and Queensland in Australia or from RMIT University culture collection.

Campylobacter strains grown for PCR testing were cultured on Oxoid Columbia agar plates supplemented with 5% defibrinated horse blood and Skirrow *Campylobacter* isolation medium supplement (Oxoid SR69). The plates were incubated in a microaerophilic atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 1 day at 42 °C. *Salmonella*, *Klebsiella*, *Proteus* and *Yersinia* spp., and *E. coli* were grown on nutrient agar aerobically at 37 °C.

Cell counts were done by direct microscopic count and viable count was by serial dilution.

DNA manipulation

All methods for DNA manipulation were described previously [5, 6].

Cloning and sequencing

The *C. jejuni* DNA fragment carrying the polymorphic *Cla*I DNA site was subcloned into the sequencing vector pGEM-7Z™. The fragment carrying the polymorphic *Cla*I site (determined by DNA hybridization to known strains with both polymorphic types) was sequenced using the di-deoxy termination method [7] using the ABI *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Perkin–Elmer) according to manufacturer's instruction and analysed using an ABI 373 automated sequencer. The sequence data were analysed using the ANGIS database.

PCR reaction

DNA amplifications were carried out using a Thermal Cycler (Perkin–Elmer) using the following reaction mixture and conditions unless otherwise specified: 2 ng of purified DNA or 2 μl of 1/10 dilution of crude cell lysate, 0·2 mM dNTP and 3·2 pmol of each primer in standard reaction buffer with [$MgCl_2$] at 2 mM and 0·1 units of Amplitaq Gold (Perkin–Elmer). The cycling protocol was: hot start at 94 °C for 5 min, then 94 °C for 30, 55 °C for 60 s, 72 °C for 2 min for a total of 35 cycles, then 72 °C for 2 min. The crude cell lysates were prepared by boiling. Specific primers were: forward primer Cjej2 (5′-GAG CAA TGC CCA AAC TGG AC-3′) and reverse primer Cjej1 (5′-TCA GGA GCA AAA GAT GAG GG -3′), The internal control primers were modified from Eyers et al, [8]: Forward primer Therm1.1(5′-TAT TCC AAT ACC AAC ATT AGT-3′) and reverse primer Therm2.1 (5′-GAA GAT ACG GTG CTA TTT TG-3′). Cjej1 and Cjej2 primers are specific for *C. jejuni* and amplify a 749 base pair (bp) fragment. Therm1.1 and Therm1.2 amplify a 306–309 bp fragment of the 23s rRNA gene of thermophilic *Campylobacter* spp.

Preparation of DNA from cell lysates and faecal material for PCR

Crude cell lysates were prepared by the boiling method. A loopful of cultured bacteria were re-suspended in 500 μl of distilled water and boiled for 10 min followed by centrifugation to remove cell debris. All strains isolated from chickens and humans were prepared for assessment by the multiplex PCR, by the boiling method. In addition 20 strains were assessed again using samples prepared by the method of Lawson et al. [9] with identical results. Faecal material and material from chicken skin samples was treated as described by Boom et al. [10] and Lawson et al. [9]; 20 samples were assessed by each method. The final pellet was re-suspended in sterile distilled water and stored at −20 °C.

RESULTS

Cloning and sequencing of the *Cla*I polymorphic region

A total of 1800 bp of *C. jejuni* DNA surrounding the *Cla*I polymorphic site was sequenced and potential PCR primer sites were selected with respect to the position of the polymorphic *Cla*I site and the G+C

https://doi.org/10.1017/S0950268801005763 Published online by Cambridge University Press

101 AGGCATTACA CAAATTTACC TAGTTCAGTT TCAACAGCAA ACAATGAATGC TGTAATAGG TTC **CGAGCAA TGCCCAAACT GGAC**TAGATA GTCTTTTAAA

201 TGGTGCCAAT TTAGATACTG CGAAATCTAT ACTTACAAAT ATTGATTTTGCA TCCTTAGG CTACAGTAGC AAAAATCCAC TTGATATGAA TACAGATGAA

301 CTTCAACAAC TTGTAAGTGA AGATGGTTTT TTTGGAGTGG AAAATACAGCC AATCGCATC GCGGATTTTG TTATTAAAGG CGGTGGCGAC GATGTTGAAA

401 AATTGAAAAA AGGTCTTGAA GGTATGAAAA AAGGATTTGA GCAAGCAGAA AAAATGTGGG GTGGAGAACT TCCTCAAATC AGTCAAAATA CAATAGATGC

[*Cla*I]

501 GGCACTTAAA AAAGTTAGCG ATCGCATTGA TGAACTAGGC GGAAAAACTCTAGATCTTCA AGCT**TAACTA** AAAATGCTTA GAAATTTCTA AGCATTTTTA

601 GTTGTTATTA ATATCAAAAG TAAAAAATTC ACTCTTAAAA TTAAAATTTCTAAGCATTTT GAGATA TCGCAGCTTC ATAGTTCTTTACTTCTTGAT ACAA

701 CATACC AAGAGCTGCT TTACTCTCTT TGTTATTTTTATCTGTAAGT TTAGAAAGCT GCAAAAGCGC TATAGCTGAA TTTGGATTAT TTGCACCTAC GGCT

801 GCTACG GCTGCTAAAA ATAAAGTTTT AGTATCTTTT GCTCCATAAT CATCAATCAA AGAATTATAA AGCGCATAAG CCTCCTCGTA TTGCTGTGCA AAAA

901 TATCCA AATAAGCTAA AGTTTGTAAA **ATACCCTCATCTTTTGCTCC TGA**AACATTA ATCAACTCTT TAAATTTCTG TCTTTCTAAA TTTAAAAGCC CTGCA

ATATG CATTAAATTA ACATAAAATT CCCTAGCTAT

Fig. 1. DNA sequence containing putative *Cla*I polymorphic site. Forward and reverse arrows indicate PCR primer sites.

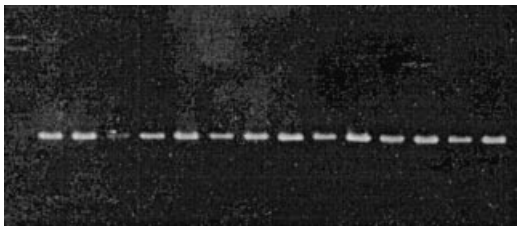

Fig. 2. Agarose gel showing 747 bp product amplified from the DNA of randomly selected *C. jejuni* strains.

content in that genomic region (Fig. 1). The two primers selected were: the forward primer Cjej1 (5′-GAG CAA TGC CCA AAC TGG AC -3′) and the reverse primer Cjej2 (5′-TCA GGA GCA AAA GAT GAG GG -3′). These were then used as described in Materials and Methods to amplify genomic DNA across the *Cla*I polymorphic region in *C. jejuni* strains.

The PCR reaction resulted in amplification of ~ 740 bp DNA fragment in all *C. jejuni* strains tested (Fig. 2 shows a selection of ~ 300 tested strains). The amplified DNA fragment could be cleaved using *Cla*I to produce a two band pattern in strains carrying the polymorphism (Fig. 3).

Specificity and sensitivity of the PCR reaction

The specificity of the PCR reaction was initially tested against enteric bacteria including *E. coli* and *Salmonella* spp as well other *Campylobacter* spp. described in Methods. Further, 10 different *C. coli* strains from identified sources (5 from humans and 5 from chickens) and 10 *C. coli* strains chosen randomly among Australian, Japanese and USA isolates as well as 20 clinical isolates were tested to ascertain specificity of the PCR assay. The DNA from other *Campylobacter* spp was not amplified using the same PCR test. As an internal control 23S RNA sequence primers which identified all thermophilic campylobacters by producing a single known PCR product of 103 bp were included (Methods). This ensures that if a negative result occurs, it is due to the lack of appropriate sequences (ie. strain is not *C. jejuni*) rather than any problem with the PCR reaction. Only *C. jejuni* strains produced the expected amplified DNA product of ~ 740 bp with the *C. jejuni* specific primers and the product could be cleaved with *Cla*I in strains carrying the polymorphic site (Fig. 4).

The specificity and sensitivity of the PCR test were also determined for crude cell lysates of *C. jejuni*, seeded faecal samples and chicken skin with surface contamination by *C. jejuni*. Chicken skin was obtained from chickens available to the public through retail outlets.

The sensitivity of the PCR test was as follows: Purified DNA could be detected at 1×10^{-9} g, which was equivalent to 120 cfu/ml as determined by viable bacterial count). The total bacterial count to give a concentration of DNA at 1×10^{-9} (calculated) was equal to 500 bacteria/ml. When the control primer was omitted from the reaction mix the detection was of even greater sensitivity with the level of detection reduced to 1×10^{-13} g, which was equivalent to 1 bacteria/2 ml.

When crude bacterial cell lysate was used, the sensitivity of detection was 3×10^4 cells/ml and for seeded faeces the level of sensitivity was 1×10^5

https://doi.org/10.1017/S0950268801005763 Published online by Cambridge University Press

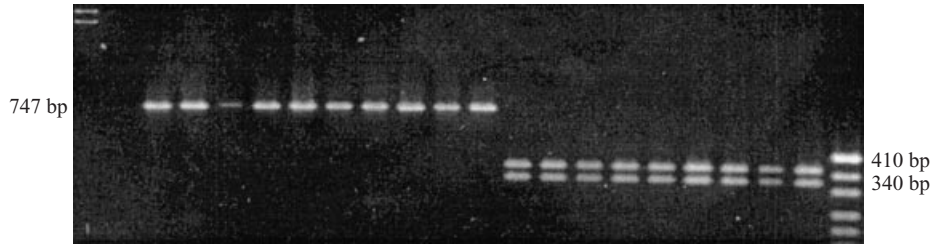

Fig. 3. Agarose gel showing 747 bp product cleaved with *Cla*I. Lanes 1 and 22 show MW marker; lanes 2–11 show *C. jejuni* strains isolated from humans and lanes 12–21 show strains isolated from chickens.

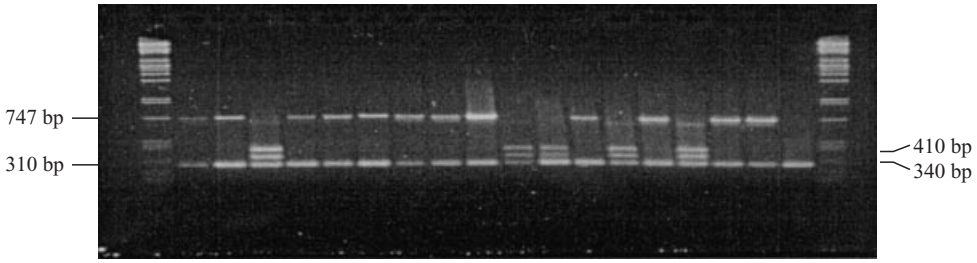

Fig. 4. Multiplex PCR profiles of *C. jejuni* strains with amplification products cleaved by *Cla*I to identify polymorphic site and 23rRNA thermophilic campylobacter identification.

cells/gm. For cells present on chicken skin – 120 cells/1ml of washing from a 1 cm² skin fragment could be detected. The PCR reaction contained 2 μl of the sample which shows that the detection level in absolute numbers with respect of total number of bacteria detected was: 1×10^2 cells for crude lysates, 1×10^3 cells in seeded faeces and 120 cells for chicken skin.

Screening of campylobacter isolates from Victoria and Queensland in Australia

Campylobacter isolates from human faeces from cases of campylobacter enteritis were screened using the PCR assay as described. All strains identified as *Campylobacter* spp other then *C. jejuni*, were further classified using the hippurate hydrolysis test. Of 129 strains from human patients from Victoria, 5 were identified as *C. jejuni* which had been misclassified as *C. coli*, 70% were of a single fragment (human type) polymorphism and 30% were of double fragment (chicken type) polymorphism. Of 91 strains from human patients from Queensland, 78 were *C. jejuni* of which 77% were of a single fragment (human type) polymorphism and 23% were of double fragment (chicken type) polymorphism. The remaining 13 strains did not react with *C. jejuni* specific primers, but did show amplified campylobacter control fragment and were subsequently identified as *C. coli* using hipurate hydrolysis test.

DISCUSSION

In this paper we report on the use of a multiplex PCR amplification of *C. jejuni* genomic DNA to yield a PCR product with a unique polymorphic site that can be used to quickly and accurately identify and group *C. jejuni* isolates from purified DNA, cell culture, skin washings and faecal samples. The test could be applied in diagnostic laboratories and can also be used in chicken flocks to detect and type colonizing *Campylobacter* spp prior to processing. The test can also be used for differentiation of *C. jejuni* from other campylobacters. The test is simple and sensitive and can detect purified DNA from a single bacterium, 10^2 *C. jejuni* cells from crude lysates, 10^3 *C. jejuni* cells in seeded faeces and 120 cells for washings from chicken skin.

A variety of methods have been reported for identification of thermophilic campylobacters using rRNA gene sequences [11–14], multiplex PCR with *gyr*A and *pfl*A genes or the *cad*F gene [15, 16]. These methods can all identify *C. coli* and *C. jejuni* and in some cases differentiate between these two related species. However, these tests have no power to differentiate types of *C. jejuni* strains. Other methods such as AFLP genotyping using *fla* genes or randomly primed DNA restriction fragments [5, 17, 18] offer great power to genotype and establish levels of relatedness of different strains for epidemiology. However these methods are unable to group or differentiate isolates from animals and humans. The

https://doi.org/10.1017/S0950268801005763 Published online by Cambridge University Press

multiplex PCR assay using the *C. jejuni Cla*I polymorphic region described in this paper is a single test that can be used to identify thermophilic campylobacters using purified DNA, cultured cells, faecal material or chicken skin and can also be used to group the majority of strains with respect to human-type or chicken-type polymorphism.

ACKNOWLEDGEMENTS

This work is supported by the Rural Industries Research and Development Corporation, Australia.

REFERENCES

1. Skirrow MB. *Campylobacter* enteritis – the first five years. J Hyg. 1982; **89**: 175–84.
2. Shane SM. *Campylobacter* infection of commercial poultry. Rev Sci Tech 2000; **19**: 376–95.
3. Blaser MJ. Epidemiologic and clinical features of *Campylobacter jejuni* infections. J Infect Dis 1997; **176**: 103–5.
4. Korolik V, Moorthy L, Coloe PJ. Differentiation of *Campylobacter jejuni* and *Campylobacter coli* strains using restriction endonuclease DNA cleavage profiles and DNA fragment polymorphisms. J Clin Microbiol 1995; **33**: 1136–40.
5. Kokotovic B, On SLW. High-resolution genomic fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* by analysis of amplified fragment length polymorphisms. FEMS Microbiol Lett 1999; **173**: 77–84.
6. Korolik V, Fry BN, Alderton MR, van der Zeijst BAM, Coloe PJ. Expression of *Campylobacter hyoilei* lipo-oligosaccharide (LOS) antigens in *E. coli*. Microbiology 1997; **143**: 3481–9.
7. Sanger F, Nicklen S, Coulsen AR. DNA sequencing with chain terminating inhibitors. Natl Acad Sci USA 1977; **4**: 5463–7.
8. Eyers M, Chapelle S, Van Camp G, Goossens H, De Wachter R. Discrimination among thermophilic *Campylobacter* spp. by polymerase chain reaction amplification of 23s rRNA gene fragments. J Clin Micro 1993; **31**: 3340.
9. Lawson AJ, Linton D, Stanley J, Owen RJ. Polymerase chain reaction detection and speciation of *Campylobacter upsaliensis* and *C. helviticus* in human faeces and comparison with culture techniques. J Appl Microbiol 1997; **83**: 375–80.
10. Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-Van Dillen PME, van der Noordaa J. Rapid and simple method for purification of nucleic acids. J Clin Microbiol 1990; **28**: 495–503.
11. Giesendorf BAJ, Quint WGF, Henkens MHC, Stegeman H, Huf FA, Niesters HGM. Rapid and sensitive detection of *Campylobacter* spp in chicken products by using the polymerase chain reaction. App Env Microbiol 1992; **58**: 3804–8.
12. Van Camp G, Fierens H, Vandamme P, Goossens H. Huyghebaert A, De Wachier R. Identification of enteropathogenic *Campylobacter* species by oligonucleotide probes and polymerase chainreaction based on 16s rRNA genes. Syst Appl Microbiol 1993; **16**: 30–6.
13. Linton D, Lawson AJ, Owen RJ, Stanley J. PCR detection, identification to species level and fingerprinting of *Campylobacter jejuni* and direct from diarrhetic samples. J Clin Microbiol 1997; **35**: 2568–72.
14. Oyarzabal OA, Wesley IV, Harmon KM, et al. Specific identification of *Campylobacter fetus* by PCR targeting variable regions of the 16S rDNA. Vet Microbiol 1997; **58**: 61–71.
15. Ragimbeu C, Salvat G, Colin P, Ermel G. Development of a multiplex PCR gene fingerprinting method using *gyr*A and *pfl*A polymorphisms to identify genotypic relatedness within *Campylobacter jejuni* species. J Appl Microbiol 1998; **85**: 829–38.
16. Konkel ME, Gray SA, Kim BJ, Garvis SG, Yoon J. Identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* based on the *cad*F virulence gene and its product. J Clin Microbiol 1999; **37**: 510–7.
17. Ayling RD, Woodward MJ, Evans S, Newell DG. Restriction fragment length polymorphism of polymerase chain reaction products applied to the differentiation of poultry campylobacters for epidemiological investigations. Res Vet Sci 1996; **60**: 168–72.
18. Duim B, Wassenaar TM, Rigter A, Wagenaar J. High-resolution genotyping of *Campylobacter* strains isolated from poultry and humans with amplified fragment length polymorphism fingerprinting. Appl Environm Microbiol 1999; **65**: 2369–75.